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TITLE: Temporal Changes in FLT3-ITD Regulation of Stem Cell Self-Renewal and Leukemogenesis

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14. ABSTRACT
My goal is to understand how mechanisms that regulate normal hematopoietic development can also influence the mutation spectra of pediatric and adult acute myeloid leukemia (AML). Genetic differences between pediatric and adult AML may underlie differences in outcomes and necessitate different treatment strategies, yet we have few insights into why these differences occur. To address this problem, we are testing whether one mutation, FLT3-ITD, differentially regulates fetal and adult progenitors. FLT3-ITD mutations are more common in adult AML than in childhood AML, and our studies to date have shown that it has age-specific phenotypes. In adult mice, FLT3-ITD depleted the hematopoietic stem cell (HSC) pool and expanded myeloid progenitor populations. These phenotypes were not evident in fetal mice, even in the presence of a collaborating *Runx1* mutation. To understand why fetal and adult progenitors responded differently to FLT3-ITD, we characterized signal transduction (e.g. STAT5 and MAPK pathways) in fetal, neonatal and adult progenitors. STAT5 was activated by FLT3-ITD at all stages of development, but MAPK was activated only in post-natal progenitors. Furthermore, STAT5 target gene regulation changed through the course of development. We are now testing whether reactivation of fetal gene products can suppress leukemogenesis. Reprogramming therapies may offer a novel approach for treating AML.

15. SUBJECT TERMS
FLT3-ITD, leukemia, hematopoietic stem cell

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2015 PROGRESS REPORT – JEFFREY A. MAGEE, M.D., Ph.D.

1. INTRODUCTION

The goal of this project is to understand why FLT3-Internal Tandem Duplication (*FLT3-ITD*) mutations cause acute myeloid leukemia (AML) more frequently in adults than in children. *FLT3-ITD* encodes a constitutively active FLT3 tyrosine kinase. This mutation occurs in ~30% of adult AML, but only 5-10% of pediatric AML and <1% of infant AML. This suggests that the mutation preferentially transforms older progenitors. To test whether FLT3-ITD has age-specific effects on hematopoietic stem cells (HSCs) and other hematopoietic progenitors, we have been characterizing its function in fetal, neonatal and adult mouse HSCs. Thus far, we have found that FLT3-ITD differentially regulates fetal and adult HSCs. Furthermore, it induces age-specific changes in signal transduction and gene expression in HSCs and more committed progenitors. We are now testing whether fetal specific gene products can re-program adult progenitors and blunt leukemogenesis. Reprogramming therapies offer a novel approach for treating AML.

2. KEYWORDS

- Flt3-Internal Tandem Duplication (FLT3-ITD)
- Hematopoietic stem cell (HSC)
- Acute myeloid leukemia
- Stat5
- MAP-kinase

3. ACCOMPLISHMENTS

Major goals:

Aim 1: To test whether *FLT3-ITD* depletes HSCs, expands restricted progenitors and promotes a myeloproliferative neoplasm during the adult, but not fetal stage of development.

Aim 2: To test whether fetal and adult hematopoietic progenitors have different FLT3-ITD driven signal transduction mechanisms and gene expression.

Aim 3: To test whether ectopic *Lin28b* expression impedes FLT3-ITD driven HSC depletion and leukemogenesis.

Accomplishments under these goals:

Aim 1: To test whether FLT3-ITD depletes HSCs, expands restricted progenitors and promotes a myeloproliferative neoplasm during the adult, but not fetal stage of development.

All of the proposed experiments related to this aim have been accomplished:

My lab used knock-in mice to test whether *FLT3-ITD* has age-specific effects on HSC self-renewal, myelopoiesis, signal transduction and gene expression. We found that *FLT3-ITD* caused a marked reduction in adult HSCs ($CD150^{+}CD48^{-}Lineage^{-}c\text{-}Kit^{+}Sca1^{+}$), consistent with prior studies, yet it had no effect on fetal HSC numbers (Fig. 1A, B). In limiting dilution transplants the mutation impaired reconstitution of the blood system by adult bone marrow, but it did not affect reconstitution by fetal liver cells (Fig. 1C, D). *FLT3-ITD* began to deplete the HSC pool by 14 days after birth in mice (Fig. 1E). Expansion of the myeloid progenitor pool and myeloproliferative neoplasms (MPN) were also evident in 14 day old *FLT3-ITD* mice but not fetal mice (Fig. 1F and not shown). *FLT3-ITD*

therefore depletes the HSC pool and induces myeloid expansion only in a post-natal developmental context.

Since *FLT3-ITD* collaborates with other mutations to drive AML, we tested whether it has age-specific phenotypes in the presence of collaborating *Runx1* mutations. Mono- and bi-allelic *Runx1* mutations are found in human *FLT3-ITD* positive AML, and they are more common in adults than children. We generated mice with *FLT3-ITD*; *Runx1* compound mutations (*Vav-Cre; FLT3*$^{ITD/+}$*; Runx1*$^{f/+}$) and analyzed HSC, multi-potent progenitor (MPP; CD48^{+}LSK) and myeloid progenitor frequencies at post-natal day (P)0, P14 and P21. We also analyzed spleen histology and blood cell morphology. We focused on the effects of compound heterozygous mutations because most *Vav-Cre; Runx1*$^{f/f}$ mice died shortly after birth, irrespective of their *FLT3-ITD* status and without evidence of leukemia (data not shown), and because *Runx1* deficiency alters HSC surface marker phenotypes. *Vav-Cre; FLT3*$^{ITD/+}$*; Runx1*$^{f/+}$ mice survived post-natally. All developed enlarged spleens with MPN by P14, and some developed AML around 3 months after birth (data not shown). The bone marrow of *Vav-Cre; FLT3*$^{ITD/+}$*; Runx1*$^{f/+}$ mice was severely depleted of phenotypic HSCs at P14 and P21, and the MPP population was expanded (Fig. 2). In contrast, newborn (P0) *Vav-Cre; FLT3*$^{ITD/+}$*; Runx1*$^{f/+}$ mice had normal liver sizes, normal HSC numbers and only a mild increase in MPPs (Fig. 2). *FLT3-ITD* therefore has distinct effects on pre- and post-natal hematopoiesis even in the setting of a collaborating *Runx1* mutation.

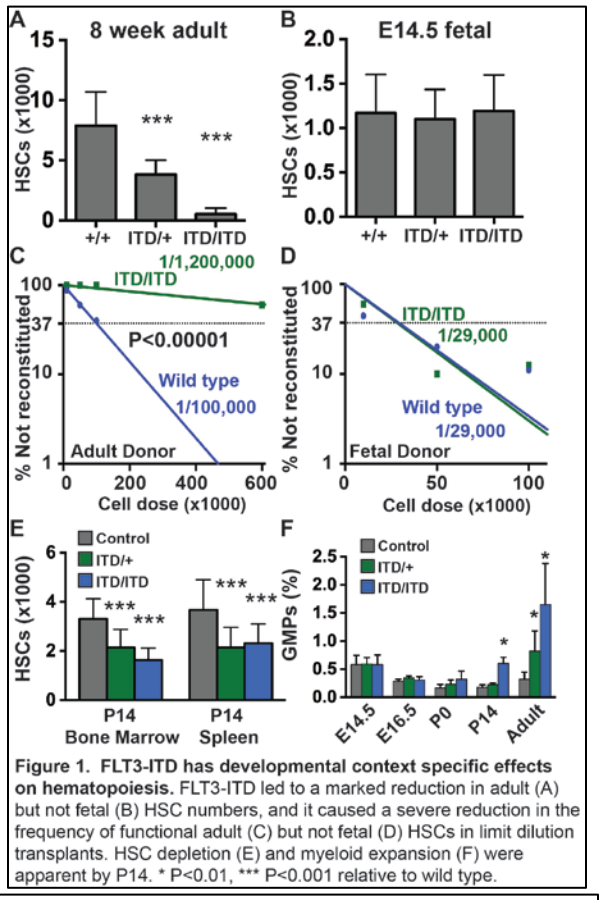

Figure 1. FLT3-ITD has developmental context specific effects on hematopoiesis. FLT3-ITD led to a marked reduction in adult (A) but not fetal (B) HSC numbers, and it caused a severe reduction in the frequency of functional adult (C) but not fetal (D) HSCs in limit dilution transplants. HSC depletion (E) and myeloid expansion (F) were apparent by P14. * P<0.01, *** P<0.001 relative to wild type.

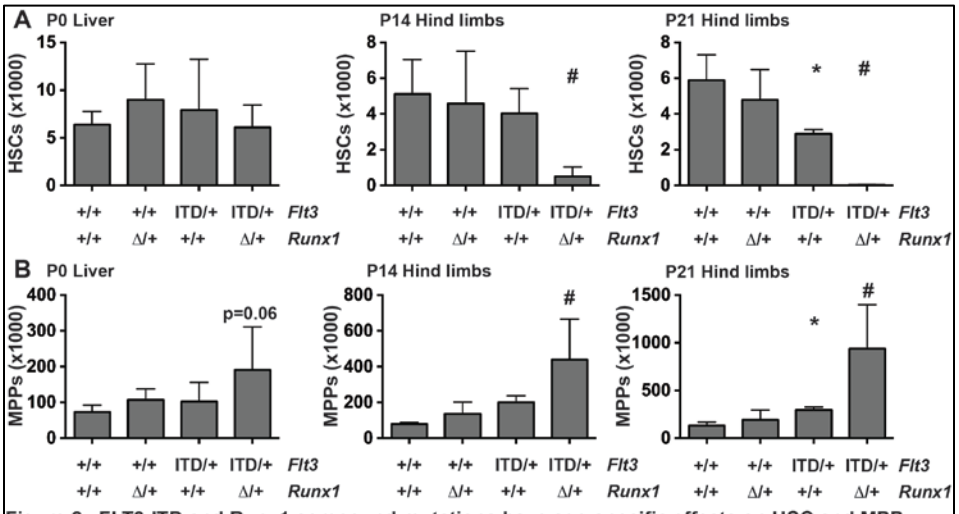

Figure 2. FLT3-ITD and Runx1 compound mutations have age-specific effects on HSC and MPP numbers. HSC numbers are reduced (A) and MPP numbers are increased in in *FLT3-ITD; Runx1* Δ/+ mice at P14 and P21 but not P0. * P<0.05 and # P<0.001 relative to wild type.

<u>Aim 2:</u> To test whether fetal and adult hematopoietic progenitors have different FLT3-ITD driven signal transduction mechanisms and gene expression.

<u>All of the proposed experiments related to this aim have been accomplished</u>:

To understand why *FLT3-ITD* has distinct effects on pre- and post-natal hematopoiesis, we evaluated signal transduction and gene expression at E14.5, P0, P14 and 8 weeks after birth. The FLT3-ITD protein has previously been shown to activate the STAT5, MAP-kinase (MAPK) and PI3-

kinase (PI3K) pathways in cultured cells. In mice, the mutation did not hyper-activate the PI3K pathway, and mTORC2 (a PI3K pathway effector) was not necessary for *FLT3-ITD* mediated HSC depletion or MPN (data not shown). In contrast, the STAT5 and MAPK pathways had distinct, age-specific activities in *FLT3-ITD* HSCs (Fig. 3A). STAT5 was hyper-activated in *FLT3-ITD* HSCs at all evaluated stages of development, but the MAPK pathway (as shown by ERK1/2 phosphorylation) was hyper-activated only in P14 and adult *FLT3-ITD* HSCs (Fig. 3A). To test whether STAT5 inactivation rescued HSC depletion and MPN, we conditionally deleted *Stat5a/b* in compound mutant *Mx1-Cre; FLT3$^{ITD/+}$; Stat5a/b$^{f/+}$* and *Mx1-Cre; FLT3$^{ITD/+}$; Stat5a/b$^{f/f}$* mice. To our surprise, *Stat5a/b* deletion exacerbated HSC depletion and myeloid progenitor expansion rather than rescuing these phenotypes (Fig. 3B, C), and this was associated with a more severe MPN (data not shown). Functional studies of the MAPK pathway are in progress, but the data already suggest that STAT5 helps to maintain *FLT3-ITD* mutant progenitors in an immature state even as other effectors promote myeloid commitment. It is now important to understand which FLT3-ITD target genes promote normal and neoplastic self-renewal, which genes remain active in fully transformed AML cells and how normal developmental programs shape the FLT3-ITD transcriptome.

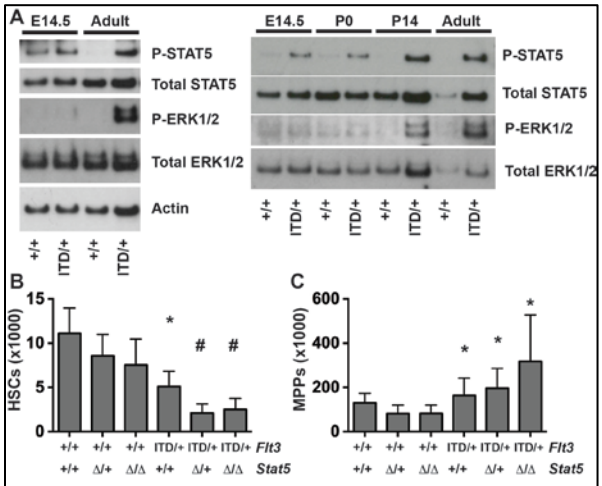

Figure 3. FLT3-ITD activated STAT5 at all ages but MAPK only in adult HSCs, and STAT5 maintained FLT3-ITD HSCs. (A) Western blots showing STAT5 and MAPK activation in control and FLT3-ITD HSCs during different stages of pre- and post-natal development. (B, C) HSC depletetion (B) and MPP expansion (C) was enhanced in FLT3-ITD mice by deleting one or both *Stat5a/b* alleles. * P<0.05, # P<0.01 relative to wild type.

To understand how FLT3-ITD target gene regulation changes with age, we used microarrays to characterize gene expression in wild type and *FLT3$^{ITD/+}$*, HSCs and MPPs at E14.5, P0, P14 and 8 weeks after birth. To determine which FLT3-ITD targets are regulated via STAT5, we also characterized gene expression in wild type, *FLT3$^{ITD/+}$*, *FLT3$^{ITD/ITD}$*, *Mx1-Cre; FLT3$^{ITD/+}$; Stat5a/b$^{f/+}$* and *Mx1-Cre; FLT3$^{ITD/+}$; Stat5a/b$^{f/f}$* adult MPPs. These experiments made several key points: 1) In wild type HSCs, most fetal-specific genes are inactivated and most adult-specific genes are activated between birth and P14. This is earlier than prior studies have suggested, and it correlates with the age at which FLT3-ITD induces HSC depletion and myeloid expansion (Fig 4A, B for representative examples). 2) FLT3-ITD does not alter gene expression until after birth, coincident with onset of the HSC depletion and myeloid expansion phenotypes (Fig. 4C). 3) FLT3-ITD target genes are more differentially expressed in MPPs than in HSCs (Fig. 4E, F), consistent with recent data suggesting that MPPs are a cell of origin for FLT3-ITD driven AML. 4) Most, but not all, FLT3-ITD target genes are STAT5 dependent (Fig. 4D). Functional studies are now necessary to identify novel, adult specific effectors of FLT3-ITD driven leukemogenesis (Aim 1) and to determine whether fetal genetic programs can suppress leukemogenesis (Aim 2). The over-arching goal is to understand whether AML cells exhibit "context addiction" (i.e. a sustained requirement for normal adult gene products and a toxic response to fetal gene products) that can be exploited therapeutically.

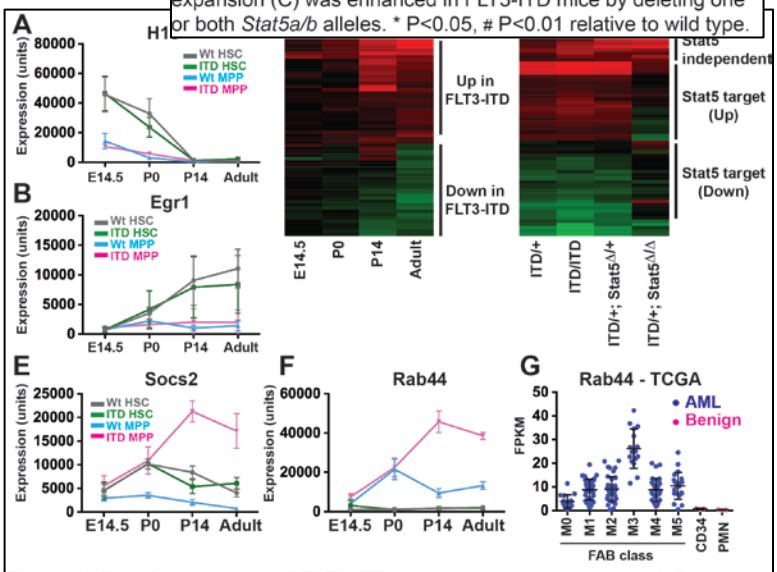

Figure 4. Developmental and FLT3-ITD target gene expression. (A,B) *H19* and *Egr1* are representative of many genes that are expressed higher (A) or lower (B) in adult HSCs as compared to fetal HSCs. Differential expression is clearly evident by P14. (C) FLT3-ITD target genes are largely activated or repressed beginning at P14. (D-F) A majority of FLT3-ITD targets are regulated by STAT5. Two examples are *Socs2* (E) and *Rab44* (F). (G) *Rab44* is an example of a FLT3-ITD target that is expressed higher in AML as compared to benign hematopoietic cells.

Aim 3: *To test whether ectopic Lin28b expression impedes FLT3-ITD driven HSC depletion and leukemogenesis.*

We are in the process of conducting these experiments as outlined in the statement of work. We have generated FLT3-ITD; iLin28b compound mutant mice and we have begun to analyze HSC frequencies in adulthood.

Opportunities for training and professional development:

This work was selected for an oral presentation at the International Society for Stem Cell Research (ISSCR) annual meeting. While at the meeting, I participated in a young investigator session as well as several additional speaker symposia. I have attended both the American Society for Hematology annual meeting and the ISSCR annual meeting in the past year. I interact with my primary mentor on this proposal, Sean Morrison, several times a year at meetings, and I plan to go to Dallas and present my work to his lab in October.

Dissemination of results to communities of interest:

Thus far, I have presented the work as an oral presentation at the ISSCR meeting. We are preparing a manuscript based on results from aims 1 and 2.

Plans for the next reporting period to accomplish the goals:

We will continue the experiments from aim 3 as outlined in the Statement of Work. This project is ahead of schedule. These plans will include further characterization of *FLT3-ITD; iLin28b* compound mutant mice and *FLT3-ITD; iLin28b; Runx1$^{FL/+}$; Ubc-CreER* mice.

4. IMPACT

Impact on the development of the principal discipline of the project:

When published, this project will provide the first evidence that leukemia causing mutations can have age-specific effects on blood forming stem cells and other immature blood cells. This likely contributes to the differences between pediatric and adult leukemias, and It raises the possibility of targeting developmental programs to suppress leukemogenesis.

Impact on other disciplines:

Nothing to report.

Impact on technology transfer:

Nothing to report.

Impact on society beyond science and technology:

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach/reasons:

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them:

Nothing to report.

Changes that had a significant impact on expenditures:

Nothing to report.

Significant changes in the use or care of human subjects:

Nothing to report.

Significant changes in the use or care of vertebrate animals:

Nothing to report.

Significant changes in the use of biohazards and/or select agents:

Nothing to report.

6. PRODUCTS

Publications:

Nothing to report.

Books or one-time publications:

Nothing to report.

Other publications:

Nothing to report.

Websites:

Nothing to report.

Technologies and techniques:

Nothing to report.

Inventions, patent applications or licenses:

Nothing to report.

Other products:

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals who have worked on this project:

Name:	Jeffrey Magee, M.D., Ph.D.
Project role	PI
Nearest person month worked	4
Contribution to Project	Dr. Magee oversees the entire research effort including the design, conduct and interpretation of experiments. He also performs experiments.
Funding Support - besides DOD	St. Baldrick's Foundation, Hyundai Hope on Wheels

Name:	Shaina Porter, Ph.D.
Project role	Post-doctoral fellow
Nearest person month worked	3
Contribution to Project	Dr. Porter designs, conducts and interprets experiments under the guidance of the PI. She has conducted the experiments related to temporal changes in FLT3-ITD regulation of HSCs and the signal transduction and gene expression studies.
Funding Support - besides DOD	Hyundai Hope on Wheels

Name:	Jenna Voigtmann
Project role	Research technician
Nearest person month worked	6
Contribution to Project	Ms. Voigtmann managed the mouse colony and assisted with experiments.
Funding Support - besides DOD	St. Baldrick's Foundation and institutional funds.

Name:	Andrew Cluster, M.D.
Project role	Clinical Fellow
Nearest person month worked	2
Contribution to Project	Dr. Cluster has characterized the FLT3-ITD; Runx1 compound mutant
Funding Support - besides DOD	Departmental support of clinical trainees- graduate medical education support.

Changes in active other support of the PD/PUI or senior key personnel:

Nothing to report.

Other organizations that were involved as partners:

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.

9. APPENDICES

None.